



**QUEEN'S
UNIVERSITY
BELFAST**

Sperm DNA damage has a negative association with live birth rates after IVF.

Simon, L., Proutski, I., Stevenson, M., Jennings, D., McManus, J., Lutton, D., & Lewis, S. (2013). Sperm DNA damage has a negative association with live birth rates after IVF. *Reproductive BioMedicine Online*, 26(1), 68-78. <https://doi.org/10.1016/j.rbmo.2012.09.019>,

Published in:
Reproductive BioMedicine Online

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
[Link to publication record in Queen's University Belfast Research Portal](#)

Publisher rights

This is the author's version of a work that was accepted for publication in Reproductive BioMedicine Online. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in Reproductive BioMedicine Online, VOL 26, 01/2013

General rights

Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

Accepted Manuscript

Article

Sperm DNA damage has a negative association with live-birth rates after IVF

L. Simon, I. Proutski, M. Stevenson, D. Jennings, J. McManus, D. Lutton,
S.E.M. Lewis

PII: S1472-6483(12)00590-1

DOI: <http://dx.doi.org/10.1016/j.rbmo.2012.09.019>

Reference: RBMO 792

To appear in: *Reproductive BioMedicine Online*

Received Date: 7 June 2012

Revised Date: 25 September 2012

Accepted Date: 27 September 2012



Please cite this article as: Simon, L., Proutski, I., Stevenson, M., Jennings, D., McManus, J., Lutton, D., Lewis, S.E.M., Sperm DNA damage has a negative association with live-birth rates after IVF, *Reproductive BioMedicine Online* (2012), doi: <http://dx.doi.org/10.1016/j.rbmo.2012.09.019>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Sperm DNA damage negatively affects live-birth rates in IVF patients

Sperm DNA damage has a negative association with live-birth rates after IVF

^{1,2*}L. Simon, ^{1*}I. Proutski, ¹M. Stevenson, ³D. Jennings, ³J. McManus, ³D. Lutton, ^{1†}S.E.M. Lewis.

¹Centre for Public Health, Institute of Clinical Sciences, Queen's University Belfast, Grosvenor Road, Belfast BT12 6BJ, Northern Ireland, UK.

² Present address: Department of Andrology, University of Utah Health Care, 675 Arapleen Dr. #205, SLC UT84108, USA.

³Regional Fertility Centre, Royal Jubilee Maternity Services, Grosvenor Road, Belfast, BT12 6BA, Northern Ireland, UK.

* Authors contributed equally

Tel: +44 (0) 28 9063 3987

Fax: +44 (0) 28 9032 8247

†To whom correspondence should be addressed. E-mail: s.e.lewis@qub.ac.uk

Abstract

Sperm DNA damage has a negative impact on pregnancy rates following assisted reproduction treatment. The aim of the present study was to examine the relationship between sperm DNA fragmentation and live-birth rates after IVF and intracytoplasmic sperm injection (ICSI). The alkaline Comet assay was employed to measure sperm DNA fragmentation in native semen and in spermatozoa following density-gradient centrifugation in semen samples from 203 couples undergoing IVF and 136 couples undergoing ICSI. Men were divided into groups according to sperm DNA damage. Following IVF, couples with <25% sperm DNA fragmentation had a live-birth rate of 33%; in contrast, couples with <50% sperm DNA fragmentation had a much lower live-birth rate of 13%. Following ICSI, no significant differences in sperm DNA damage were found between any groups of patients. Sperm DNA damage was also associated with low live-birth rates following IVF in both men and couples with idiopathic infertility: 39% of couples and 41% of men with idiopathic infertility have high sperm DNA damage. Sperm DNA damage assessed by the Comet assay has a close inverse relationship with live-birth rates after IVF.

Keywords: Comet assay, ICSI, IVF, male infertility, sperm DNA damage

Introduction

Infertility affects ~15% of couples of reproductive age (Cates *et al.*, 1985; Hull *et al.*, 1985; Kols and Nguyen, 1997; Rutstein and Shah, 2004) with male infertility contributing to nearly 50% of all cases (Irvine, 1998; Niederberger *et al.*, 2007; Vela *et al.*, 2009; World Health Organization, 2010). As a result of population ageing and adverse changes in lifestyle, infertility continues to increase but with only marginal improvement in pregnancy and birth rates after assisted reproduction treatment in the developed world (de Mouzon *et al.*, 2010; Dupas and Christine-Maitre, 2008; HFEA, 2008; Povey AC and Stocks, 2010). In the last 30 years, assisted reproduction treatment has become increasingly utilized, with the number of cycles (de Mouzon *et al.*, 2010) increasing by 4% per year in Europe. Still, pregnancy and live-birth rates remain disappointingly low (average 27–33%; de Mouzon *et al.*, 2010; HFEA, 2008). One reason for this is that little has been done to resolve the causes and potential therapies for male infertility at the molecular level. Furthermore, there are currently no routine pharmaceutical therapies for male infertility.

Sperm DNA damage has been identified as a major contributor to male infertility as well as outcomes following assisted reproduction treatment, including impaired embryo development, miscarriage and birth defects in the offspring (Evenson *et al.*, 1980, 1999; Balasuriya *et al.*, 2011; Bungum *et al.*, 2011; Ebner *et al.*, 2011; Freour *et al.*, 2010; Gu *et al.*, 2011; Koskimies *et al.*,

2010; Simon *et al.*, 2011; Zribi *et al.*, 2011). There are a number of assays available to detect sperm DNA damage (see the ESHRE position paper by Barratt *et al.*, 2010). The Comet assay is becoming a popular method to determine sperm DNA damage. It is recognized as the most sensitive of all methods and routinely used to assess genotoxic DNA damage in somatic cells (Decordier *et al.*, 2010; Dhawan *et al.*, 2009; Jha 2008; Pfuhler *et al.*, 2005). It is the only technique that allows quantification of DNA damage in individual cells, so it is particularly useful in a heterogeneous cell population like spermatozoa. The Comet assay measures both single- and double-strand breaks using an alkaline pH method (Collins *et al.*, 2008; Villani *et al.*, 2010).

The present study employed the alkaline Comet assay to examine sperm DNA damage in patients undergoing IVF and intracytoplasmic sperm injection (ICSI). The relationship between sperm DNA fragmentation and pregnancy, miscarriage and live-birth rates was examined. The second objective of this study was to assess the incidence of sperm DNA damage in couples and male partners with idiopathic infertility and determine if there was a relationship between their diagnosis and their treatment outcomes. Thirdly, this study determined the repeatability of the alkaline Comet assay by comparing data from three or two replicate slides for the same patient.

Materials and methods

This project was approved by the Office for Research Ethics Committees in Northern Ireland and the Royal Group of Hospitals Trust Clinical Governance Committee (ref. no. 06/NIR01/142, 9 January 2007). The study was conducted at the Regional Fertility Centre, Royal Jubilee Maternity Services, Belfast, Northern Ireland, UK. Sperm samples for research were obtained after written consent given by each couple.

Study population

A total of 203 couples undergoing IVF (age 35.6 ± 0.3 years, mean \pm standard error) and 136 couples undergoing ICSI (age 34.9 ± 0.4 years) were recruited in the study after a minimum of 1 year of unprotected intercourse without pregnancy.

Of the 203 men undergoing IVF, a subset was identified as the unexplained group ($n = 147$), which consisted of those remaining after the exclusion of men diagnosed with infertility according to criteria from the World Health Organization (2010; low sperm count, reduction in motility and normal morphology) or with a known history of fertility problems including blockages, testicular cancer or abnormal development of reproductive organs. For couples with idiopathic infertility ($n = 70$), those with women with endometriosis, ovulation dysfunction, hormone imbalance, ovarian cysts or tumours were also excluded.

Semen analysis and sperm preparation

Semen samples were provided by masturbation by the male partner after 2–5 days of recommended abstinence. After liquefaction, routine semen analyses were performed according to guidelines from the World Health Organization (2010) and subsequently semen was prepared using a two-step discontinuous Puresperm gradient (90–45%; Hunter Scientific, UK). Two populations of spermatozoa for each patient were used to measure DNA damage by Comet assay: the whole population (native semen) and those prepared by density-gradient centrifugation, typical of those used for clinical treatment.

Assisted reproduction treatment

All IVF cycles were performed according to routine procedures (Donnelly *et al.*, 1998). Briefly, ovulation induction was achieved with recombinant FSH following a long protocol of pituitary desensitization with a gonadotrophin-releasing hormone analogue. Human chorionic gonadotrophin was administered when there were at least four follicles of >17 mm diameter, 36 h before oocyte retrieval. Mature, metaphase-II oocytes were obtained by vaginal ultrasound-guided

aspiration and cultured in media (G5 sequential media series; Vitrolife, Goteborg, Sweden) at 37 C with 6% CO₂ in air. The ICSI procedure has been described in detail previously (Van Steirteghem, *et al.*, 1993). In brief, a suspension of washed spermatozoa was placed in polyvinylpyrrolidone (Vitrolife) and a free, motile spermatozoon was immobilized. The spermatozoon was aspirated into the injection pipette tail-first and injected into an oocyte. Fertilization was recorded 12–16 h after injection. In each case, one or two embryos were transferred into the uterine cavity after an additional 24–48 h. Luteal-phase support was provided by vaginally administered progesterone. An intrauterine pregnancy with fetal heart beat was confirmed by ultrasound 5 weeks after embryo transfer.

Alkaline Comet assay

Sperm DNA fragmentation was assessed using single-cell gel electrophoresis (Comet) assay which has been previously optimized for human spermatozoa (Donnelly *et al.*, 1999; Hughes *et al.*, 1997). Briefly, fully frosted slides were covered with 150 μ l of 1% normal-melting-point agarose and left to solidify for 15 min at room temperature. Sperm samples were diluted to a final concentration 2×10^6 cells/ml with phosphate-buffered saline and 10 μ l cell suspension were mixed with 75 μ l of 0.5% low-melting-point agarose and placed on slides. After 15 min, slides were immersed in cold lysis solution (2.5 mol/l NaCl, 10 mmol/l Na₂EDTA, 10 mmol/l Tris and 1% Triton X-100) and incubated for 1 h. Following lysis, slides were incubated with 10 mmol/l dithiothreitol for 30 min at 4 C followed by incubation with 4 mmol/l lithium diiodosalicylate for 90 min at room temperature. Slides were incubated in cold electrophoresis buffer (300 mmol/l NaOH, 1 mmol/l EDTA, pH 13) for 20 min to allow DNA to unwind. Electrophoresis was carried out at 25 V, 300 mA for 10 min. Slides were flooded with three changes of neutralization buffer (0.4M Tris) for 5 min. Slides were stained with 20 μ g/ml ethidium bromide and visualized using Komet version 5.5 (Andor Technology, Belfast, UK). Fifty comets were analysed for each slide.

Statistical analysis

Since some of the groups contained a small number of patients, residuals were tested with Statistical Package for the Social Sciences for Windows version 15 (SPSS, Chicago, IL, USA) to assure normality of distribution so that t-testing was appropriate. Having confirmed that residuals followed a normal distribution, *P*-values were calculated using an unpaired two-tailed t-test (GraphPad Prism for Windows version 5.01; GraphPad Software, San Diego, California, USA). Data are presented as mean \pm standard error.

The chi-squared test was used to assess whether there was a linear trend between the level of sperm DNA damage and pregnancy or live-birth rates in IVF and ICSI patients as well as men and couples with idiopathic infertility. Pregnancy and live-birth rates were organized in the frequency tables according to the level of sperm DNA damage and chi-squared and *P*-values were obtained for each analysis.

Repeatability assessment

Analysis of repeatability was performed according to the guidelines of ISO 5725 (1994) to determine the repeatability and reproducibility of a standard measurement method. Fifty-two patient samples were prepared in duplicate (28/52) or in triplicate (24/52) (128 measurements in total). Five comets were scored on 10 different areas of each slide (50 comets per slide). Data from 50 comets for each measurement were used to calculate means and standard errors. Residual values were calculated to identify and remove outliers using Dixon's test. The test statistic r_{22} was calculated using the equation $r_{22} = \frac{X_3 - X_1}{X_{n-2} - X_1}$ and compared with the critical value for r_{22} at a

significance level of 0.05. The repeatability (*r*) was defined as $r = 2.8 \sqrt{S_r^2}$, where S_r^2 is the repeatability variance or average of the within-laboratory variances and calculated as

$S_r^2 = \frac{\sum_{i=0}^n S_i^2}{n}$. The S_r^2 values were calculated separately for duplicate and triplicate measurements based on 75 degrees of freedom.

Results

Demographic characteristics of patients undergoing IVF

Patients receiving IVF were divided in three groups according to the treatment outcome: (i) clinical pregnancy that resulted in miscarriage; (ii) clinical pregnancy that resulted in a live birth; and (iii) a nonpregnant group. Each group was compared with the live-birth group and confidence intervals and *P*-values were calculated for each comparison.

When the miscarriage group was compared with the live-birth group, the only parameter that differed significantly was the total embryo cumulative score (**Table 1**). Between nonpregnant couples and the live-birth groups, there were significant differences in female age (35.9 ± 0.3 versus 34.5 ± 0.7 years, $P = 0.04$), male age (37.5 ± 0.4 versus 35.5 ± 0.9 years, $P = 0.02$), transferred embryo cumulative score (35.8 ± 1.7 versus 43.0 ± 2.7 , $P = 0.01$) and the number of fertilized oocytes (4.4 ± 0.3 versus 6.9 ± 0.6 , $P < 0.0001$).

Demographic characteristics of patients undergoing ICSI

Patients undergoing ICSI were divided into three groups similarly to those undergoing IVF (**Table 2**) and each group was compared with the live-birth group. No statistically significant difference was detected between the miscarriage and live-birth groups in any parameters studied. However, there was a significant decrease in number of fertilized oocytes in nonpregnant couples compared with the live-birth group (4.9 ± 0.3 versus 6.1 ± 0.6 , $P = 0.04$).

Sperm DNA fragmentation and treatment outcomes following IVF and ICSI

The level of sperm DNA damage was significantly higher for groups undergoing IVF in the nonpregnant group compared with the live-birth group both in terms of native semen and those prepared by density-gradient centrifugation ($P < 0.01$ and $P < 0.05$, respectively, **Figure 1**). In contrast, no significant difference was found in DNA damage between any of the groups undergoing ICSI in both native and prepared spermatozoa (**Figure 1**).

Patients undergoing IVF were divided into three groups depending on the level of sperm DNA damage (0–25%, 25–50% and over 50%) in accordance with previously reported clinical thresholds for achieving successful pregnancy after natural conception, IVF and ICSI, respectively (Simón *et al.*, 2011). This previous study reported that if men had <25% sperm DNA damage, they were more likely able to achieve a pregnancy without assisted reproduction treatment. If the men had 25–50% sperm DNA damage, their chances of success following IVF were high. However, if the men had >50% sperm DNA damage, their best chance of success was with ICSI. The proportions of pregnant couples, couples with miscarriages and couples whose pregnancies resulted in live birth were also calculated for each group (**Table 3**). The highest proportion of pregnant couples (39.4%) was recorded in the group of patients with <25% sperm DNA damage. More importantly, 11 out of the 13 pregnancies in this group of patients (33.3% of the whole IVF group) resulted in live birth. When the sperm DNA fragmentation threshold was increased to 25–50%, the proportions of pregnancies and live births declined to 29.6% and 23.9%, respectively. Statistically significant reductions in pregnancy rate (chi-squared = 8.05, $P = 0.005$) and live-birth rate (chi-squared = 7.20, $P = 0.007$) were also recorded in patients with >50% sperm DNA damage (16.2 and 13.1%, respectively).

Analysis of pregnancy and live-birth rates in patients undergoing ICSI showed that there was no relationship between sperm DNA fragmentation and either pregnancies or live births (**Table 3**).

Further, no relationship was found between sperm DNA damage and child gender or the number of singletons compared with twins (data not shown).

Sperm DNA fragmentation in men with normal semen analysis (idiopathic infertility)

To gain further insight into the relationship between different types of infertility and treatment outcomes, this study assessed DNA fragmentation levels in couples undergoing IVF according to diagnosis. Patients were divided into two groups depending on infertility diagnosis: (i) idiopathic men (couples with a diagnosed female factor but no detectable problems in the male partners); and (ii) idiopathic couples (no detectable male or female factors). Sperm DNA fragmentation was assessed in each group and related to pregnancy, miscarriage and live-birth rates. Sperm DNA damage in the idiopathic men group ($n = 147$) was $46.9 \pm 1.8\%$ in native semen and $32.8 \pm 1.6\%$ in prepared spermatozoa (**Table 4**). The overall pregnancy and live-birth rates in this patient group was 21.1% (31 out of 147 patients) and 17.0% (25 out of 147 couples), respectively (**Table 5**).

Using 25% DNA damage as a cut off to predict successful pregnancies following IVF (Simon *et al.*, 2011) 23 (15.6%) patients had <25% DNA damage in native semen. Sixty-four men (43.5%) had 25–50% DNA fragmentation while 60 men (40.8%) had >50% sperm DNA damage. The pregnancy rate for men with <25% sperm DNA fragmentation was 26.1%. All couples in this group had successful pregnancies. While a slight decrease in pregnancy rate was detected for the group of patients with 25–50% sperm DNA fragmentation (25.0%), the live-birth rate dropped markedly to 18.8%. The pregnancy rate in patients with >50% sperm DNA fragmentation was 15.0% while the live-birth rate in this group was 11.7%. This was a more than 2-fold reduction compared with the live-birth rates in the <25% sperm DNA damage group. The chi-squared test, however, did not show statistical significance of the trend (chi-squared = 2.69).

Sperm DNA fragmentation in couples diagnosed with idiopathic infertility

The same analysis was performed for 70 couples with idiopathic infertility (no detectable cause of male or female infertility). The DNA damage levels in native and prepared spermatozoa did not differ significantly from that of the group of men with normal semen parameters and were 45.8 ± 2.6 and 32.7 ± 2.4 , respectively (**Table 4**). The same proportion of patients had <25% sperm DNA damage (15.7%) and 25–50% sperm DNA fragmentation (45.7%, **Table 5**). Slightly lower numbers of couples with idiopathic infertility (38.6%) had >50% sperm DNA damage compared with men with idiopathic infertility whose female partners had detectable causes.

Lower overall pregnancy and live-birth rates were recorded in this group (20.0% and 14.3%, respectively). The pregnancy and live-birth rates in the group with <25% sperm DNA damage were both 27.3% (**Table 5**). The pregnancy rates for the other two groups of patients decreased to 21.9% and 14.8% for 25–50% sperm DNA damage and >50% sperm DNA damage, respectively. Surprisingly low live-birth rates (12.5% and 11.1%) with a concomitant increase in miscarriage rates were recorded for both groups compared with patients with <25% sperm DNA fragmentation; however, the trend was not statistically significant (chi-squared = 1.24). Subdividing patients into subgroups with 50–60%, 60–70%, 70–80% and 80–90% sperm DNA damage showed a similar distribution pattern between the groups (data not shown).

Repeatability of the Comet assay

Repeatability refers to within-laboratory agreement between replicate observations of the same test performed by the same operator under similar conditions. Repeatability of Comet assay was assessed through comparison of duplicate or triplicate results from the same sample. One-way ANOVA was used to calculate variance. Based on this value, the standard errors of precision for single, duplicate and triplicate measurements were measured. To conduct the repeatability assessment of the alkaline Comet assay, DNA damage data was obtained from five random comets scored on 10 different areas on each slide (50 comets per slide) on two or three replicate slides for

all 52 samples. Data from 50 comets were used to calculate mean and standard deviation, which provided 128 means and 76 degrees of freedom. After plotting residual values for mean DNA damage, three potential outliers were detected in this dataset and Dixon's test was used for identification and rejection of the outliers (**Figure 2**). One of them was classified as an outlier ($P = 0.05$) after applying Dixon's test and was excluded from further analysis, thus decreasing the degrees of freedom to 75.

The S_r^2 value for a single DNA damage measurement was found to be 3.73% while it decreased to 2.65% and 2.16% for duplicates and triplicates, respectively. According to the standard error of precision, the likely range of the true value for a patient with 60% sperm DNA damage will be narrow, at 55.7–64.3%.

Discussion

Sperm quality is routinely assessed by measuring concentration, motility and morphology (World Health Organization, 2010). While conventional semen analysis can be useful as initial evaluation of a patient's fertility status, there is growing evidence that it has limited value in diagnosing male infertility or predicting success of assisted reproduction (Berlinguer *et al.*, 2009; Björndahl and Haugen, 2008; Ford, 2010; Garrido *et al.*, 2008; Nagler, 2011; Saleh *et al.*, 2003; Shamsi *et al.*, 2010). Although currently not in the battery of routine clinical tests offered to patients, sperm DNA damage has been demonstrated to be a promising tool in determining the male patient's fertility status and to predict successful treatment in terms of the short-term goals of fertilization and pregnancy and the long-term goal of healthy offspring.

No methods are currently available to measure DNA damage in spermatozoa that will be subsequently used clinically. However, of the methods currently available to determine sperm DNA damage, the Comet assay is considered the most sensitive (Irvine *et al.*, 2000; Leroy *et al.*, 1996; Villani *et al.*, 2010). It detects single- and double-strand breaks as well as abasic sites (Villani *et al.*, 2010). It is the only test that quantifies DNA fragmentation in an individual spermatozoon. In addition, it requires a small number of cells, therefore making it suitable to assess DNA damage in semen samples supplied for routine analysis without the need of a further sample dedicated to DNA testing. Most importantly, robust clinical threshold values have now been established for this test (Simon *et al.*, 2011). It has also been demonstrated to provide a stronger prognostic ability to predict fertilization after IVF than progressive motility (Simon and Lewis, 2011). Some have suggested that the low number of cells (50 spermatozoa in duplicate or triplicate) counted in the Comet assay gives it low repeatability. In order for it to be fully accepted by the clinicians and scientific community, there must be tight regulations on the protocol used. Furthermore, to be approved as a standard analytical tool in clinical laboratories, the Comet assay must be shown to have single laboratory validation, the process that includes optimization, standardization, establishing of thresholds, measuring diagnostic specificity and sensitivity and calculating repeatability and reproducibility. This study group has reported results for optimization and standardization of the Comet assay together with clinical thresholds, diagnostic specificity and sensitivity (Lewis and Simon, 2010; Simon *et al.*, 2011). The current study evaluated within-laboratory variance (repeatability) of the assay as recommended in ISO 5725 and found results highly repeatable and consistent between replicate slides. The standard error of precision for duplicate slides was found to be 2.65%. The greatest source of error in detecting tail DNA in the Comet assay is the electrophoresis step, in particular the drop in voltage across the gel (Collins *et al.*, 2008). To account for that, the current study routinely analysed five comets on 10 different areas of the slide, thus increasing sample representativeness, and this was also reflected in extremely low variability between the duplicate or triplicate slides.

This study observed a close negative relationship between nonpregnant and the live-birth groups in some parameters of treatment outcome, in particular total embryo cumulative score and transferred embryo score and a failure to achieve a pregnancy following IVF. This is consistent with previous reports from this study group (Simon *et al.*, 2011) and others (Hu *et al.*, 1998; Lee *et al.*, 2006; Loi *et al.*, 2008; Qian *et al.*, 2008; Sjöblom *et al.*, 2006). Both the total embryo

cumulative score and transferred embryo cumulative score were considerably reduced in the nonpregnant group compared with patients having live births after IVF. In contrast, in ICSI patients, no relationship was detected in either total or transferred embryo cumulative score and live births. These results are in agreement with other studies that have shown a strong correlation between embryo quality (Avendaño *et al.*, 2010; Meseguer *et al.*, 2008) and sperm DNA fragmentation.

There is a growing body of research showing a link between high sperm DNA damage and poor outcomes, including fertilization rate, pre- and post-implantation embryo development and implantation rate (Anendano and Oehninger, 2011; Host *et al.*, 2000; Hu *et al.*, 1998; Meseguer *et al.*, 2008; Qian *et al.*, 2008; Simon *et al.*, 2011; Sjöblom *et al.*, 2006). This has also been reported for the multiparous pig where the number of piglets per litter is reduced with higher sperm DNA damage (Ballachey *et al.*, 1988). In another study where female pigs had proven fertility, the negative influence of sperm DNA damage on reduced pregnancy outcomes was also evident (Didion *et al.*, 2009).

The main focus of the present study was to determine, as far as is known for the first time using the Comet assay, if there was an association between levels of sperm DNA damage in male patients and live-birth rates following IVF and ICSI. As with this study group's earlier results of total embryo cumulative score, transferred embryo cumulative score and number of oocytes fertilized (Simon *et al.*, 2010), the inverse relationship between sperm DNA damage and outcome was consistent for both native semen and prepared spermatozoa. Furthermore, analysis of pregnancy rates and sperm DNA damage in IVF patients showed a strong negative relationship, with pregnancy rates declining from 39.4% in men with <25% sperm DNA damage to 16.2% in patients with >50% sperm DNA fragmentation. This, again, is well supported by this study group (Simon *et al.*, 2011) and those of others (Bakos *et al.*, 2008; Benchaib *et al.*, 2007; Bungum *et al.*, 2007; Zini, 2011).

A relationship between high sperm DNA fragmentation and male infertility is well documented in the literature (Evenson *et al.*, 1980; Aitken and Curry, 2011; Mahfouz *et al.*, 2010; Simon *et al.*, 2011). In the current study, the relationships between sperm DNA fragmentation and outcomes, including live-birth rates in couples with idiopathic infertility (all couples with either male and female factors were excluded) undergoing IVF, were also assessed. Elevated levels of sperm DNA damage in both native and prepared spermatozoa in comparison to the low levels of sperm DNA damage ($12.47 \pm 1.67\%$) reported in native spermatozoa of fertile donors (Simon *et al.*, 2011) were found in the couples with idiopathic infertility.

While overall pregnancy rates for IVF and ICSI were found in line with those reported recently for Europe (de Mouzon *et al.*, 2010), they declined to 21% for both men and couples with idiopathic infertility. Since it is well recognized that pregnancy rates after IVF and ICSI are much lower compared with pregnancy rates after natural conception and need to be improved (Habbema *et al.*, 2009; Tam *et al.*, 2005), a further reduction in pregnancy rates in groups of patients with idiopathic infertility is an unacceptable and worrying fact. Obviously, efforts should be concentrated on elucidating the causes of idiopathic infertility in order to prescribe appropriate and effective treatment for these patients.

Of couples undergoing IVF, 72% of men and 34.5% had idiopathic infertility. Over 40% of men (60 out of 147) and nearly 39% of couples (27 out of 70) with idiopathic infertility had sperm DNA damage >50%. If these levels of damage are compared with those reported in spermatozoa of donor men with proven fertility by this study group (Simon *et al.*, 2010), the current results suggest that sperm DNA damage is the cause of infertility in a substantial number of men. Another previous study (Simon *et al.*, 2011) showed that 50% sperm DNA damage is the clinical threshold above which men are less likely to achieve a pregnancy by IVF. The live-birth rates are depressingly low (11.7% and 11.1%). Furthermore, if these couples or men are removed from the patients receiving IVF, the success rates in terms of pregnancies and live-birth rates will increase enormously; they would be expected to have high success rates since no anomalies can be detected.

It is now clear that many men with normal semen parameters have high levels of sperm DNA damage. They are often mistakenly classified as having idiopathic infertility or even being fertile individuals which, in turn, can lead to offering intrauterine insemination or IVF to such couples with little chance of success. This fact is reflected in disappointingly low effectiveness of IVF, which is measured as the cumulative incidence of live delivery after commencing IVF (Stewart *et al.*, 2011).

Since many women will not return after a poor response to IVF, this value may overestimate the cumulative live-birth rate, so that the actual live-birth rates following IVF are even smaller. Yet, it falls to an unprecedented low of 11.7% and 11.1% in men and couples diagnosed with idiopathic infertility who have >50% sperm DNA damage, respectively. According to sperm DNA fragmentation analysis these patients should be directed straight to ICSI, thus avoiding loss of valuable biological time, cost of failed cycles and heartache after repeatedly unsuccessful cycles of IVF. This once again underscores the importance of DNA fragmentation analysis in guiding the patients to the appropriate treatment and necessity of including it in routine clinical testing (Bungum *et al.*, 2007; Giwercman *et al.*, 2009).

In agreement with a previous report published by this study group (Simon *et al.*, 2010), this study found that spermatozoa from patients having ICSI that did not result in clinical pregnancy tended to have a higher level of DNA fragmentation but this was not significantly greater than that of the live-birth group irrespective of type of spermatozoa (native or prepared) used in the assay. It has been reported in the literature that sperm DNA damage assessment may be less informative in patients undergoing ICSI since this technique bypasses all natural selection barriers such as binding to the zona pellucida, acrosomal reaction and fusion to the ooplasm, and therefore fertilization with highly DNA fragmented spermatozoa is still possible (Avendano and Oehninger, 2011; Zini *et al.*, 2011).

There is a striking decrease in the live-birth rates following IVF in couples with high sperm DNA damage and to a lesser degree after ICSI. Live-birth rates decreased by more than 2-fold, from 33.3% to 13.1% (**Table 3**) in IVF patients with high (>50%) sperm DNA fragmentation compared with <25% sperm DNA damage. Not surprisingly, the number of ICSI patients with <25% sperm DNA damage was very small (only nine out of 136 patients), so the group was too small to determine any associations between levels of sperm DNA damage and live-birth rates. However, it is worth noting that both pregnancies in this group of patients resulted in live births. In agreement with a previous report from this group (Simon *et al.*, 2010), this study observed a shift towards a higher level of sperm DNA damage as well as an increase in average sperm DNA fragmentation in ICSI patients compared with IVF patients. Since ICSI is a more efficient treatment, in particular for patients with higher sperm DNA fragmentation levels, this resulted in slightly greater live-birth rates after ICSI compared with IVF for groups with the same levels of sperm DNA damage. A strong decline in live-birth rates, from 32.4% to 20.4%, was detected in ICSI patients with 25–50% sperm DNA damage compared with those with >50% sperm DNA damage. This contradicts the finding of Bungum *et al.* (2007) that delivery rates after ICSI rose from 35.3% to 42.4% for patients with DNA fragmentation <30% and >30%, respectively. This may be attributed to a different technique used in the study, since the sperm chromatin structure assay measures the proportion of spermatozoa with fragmented DNA in the ejaculate rather than DNA damage per spermatozoon, as assessed here by the alkaline Comet assay. One question that comes to mind is why do spermatozoa with DNA damage not reduce success following ICSI? There are at least four hypotheses as to why this is, as discussed below.

First, up to 30% of women having ICSI have no detectable problems. They may be fertile and their oocytes may have more capacity to repair DNA damage even if the injected spermatozoon is of poor quality. This is supported by the findings of Meseguer *et al.* (2011), where high-quality oocytes from donors offset the negative impact of sperm DNA damage on pregnancy. Further, the study of Devroey *et al.* (1996) showed that significantly fewer good-quality embryos were available for transfer from women older than 40 years compared with the younger group, reflecting better DNA repair capacity of oocytes from young women. This is relevant in that women undergoing ICSI are often younger than those undergoing IVF as their cause of infertility

has been diagnosed earlier and they have not spent time undergoing failed intrauterine insemination or IVF before embarking on a cycle of ICSI.

Second, in ICSI, the gametes are not subjected to prolonged culture, so the spermatozoa may have less damage than those exposed to culture media overnight, as in IVF. The recent major study from Dumoulin *et al.* (2010) shows that even the birthweight of IVF babies can be markedly influenced by minor differences in culture conditions. In contrast to IVF, ICSI spermatozoa are injected into the optimal environment of the oocyte within a few hours of ejaculation. This may protect them from laboratory-induced damage.

Third, much sperm DNA damage is caused by oxidative stress (Aitken *et al.*, 2010) and so these spermatozoa are producing reactive oxygen species. If they are used in IVF, the oocyte may be exposed to oxidative assault during the overnight incubation from these ~0.5 million spermatozoa. In ICSI, the oocyte is protected from this attack and can use its energies to repair the damage in the spermatozoon immediately following fertilization.

Fourth, there is now evidence that embryos created from spermatozoa with high DNA damage are associated with early pregnancy loss (reviewed by Zini *et al.*, 2008, Robinson *et al.*, 2012) so ICSI success rates are sometimes affected adversely by sperm DNA damage, but at a later stage.

In conclusion, this study found a strong negative association of sperm DNA fragmentation levels and live-birth rates in IVF patients. The relationship was more pronounced in patients with idiopathic infertility, suggesting that a possible cause of idiopathic infertility might be a high degree of sperm DNA fragmentation in such patients. In addition, this study demonstrates an importance and feasibility of sperm DNA damage testing to not only assess the treatment outcomes in general, but also to specifically evaluate live-birth rates. Lastly, determination of sperm DNA fragmentation is highly repeatable and robust. It provides additional information to help clinicians to tailor assisted reproduction treatment for men with sperm DNA damage to that particular couple's needs.

Acknowledgements

The authors gratefully acknowledge the funding of Hamilton Thorne Biosciences Ltd and Ulster Garden Villages.

References

- Aitken, R.J., De Iuliis, G.N., 2010. On the possible origins of DNA damage in human spermatozoa. *Mol Hum Reprod.* 16, 3–13.
- Aitken, R.J., De Iuliis, G.N., Finnie, J.M., Hedges, A., McLachlan, R.I., 2010. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Hum Reprod.* 25, 2415–2426.
- Aitken, R.J., Curry, B.J., 2011. Redox Regulation of Human Sperm Function: From the Physiological Control of Sperm Capacitation to the Etiology of Infertility and DNA Damage in the Germ Line. *Antioxid Redox Signal.* 14, 367–381.
- Aravindan, G.R., Bjordahl, J., Jost, L.K., Evenson, D.P., 1997. Susceptibility of human sperm to in situ DNA denaturation is strongly correlated with DNA strand breaks identified by single-cell electrophoresis. *Exp Cell Res.* 236, 231–237.
- Avendaño, C., Franchi, A., Duran, H., Oehninger, S., 2010. DNA fragmentation of normal spermatozoa negatively impacts embryo quality and intracytoplasmic sperm injection outcome. *Fertil Steril.* 94, 549–557.
- Avendaño, C., Oehninger, S., 2011. DNA Fragmentation in Morphologically Normal Spermatozoa: How Much Should We Be Concerned in the ICSI Era? *J Androl.* 32, 356–63.
- Bakos, H.W., Thompson, J.G., Feil, D., Lane, M., 2008. Sperm DNA damage is associated with assisted reproductive technology pregnancy. *Inter J Androl.* 31, 518–526.
- Ballachey BE, Saacke RG, Evenson DP, The sperm chromatin structure assay: Relationship with alternate tests of sperm quality and heterospermic performance of bulls. *J. Andrology.* 1988;9:109–115.
- Balasuriya, A., Speyer, A.B., Serhal, P., Doshi, A., Harper, J.C., 2011. Sperm chromatin dispersion test in the assessment of DNA fragmentation and aneuploidy in human spermatozoa. *Reprod Biomed Online.* 22, 428–436.

- Christopher L.R. Barratt, R. John Aitken Peter de Boer, Lars Björndahl, Douglas T. Carrell, Sally D. Perreault, Ulrik Kvist, Sheena E.M. Lewis, Melissa J Perry, Liliana Ramos, Bernard Robaire, Steven Ward and Armand Zini. Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications. A position report. *Human. Reproduction*. 2010 25: 824–838; doi:10.1093/humrep/dep465. <http://humrep.oxfordjournals.org/cgi/content/abstract/25/4/824?etoc>
- Benchai, M., Lornage, J., Mazoyer, C., Lejeune, H., Salle, B., Guerin, J.F., 2007. Sperm deoxyribonucleic acid fragmentation as a prognostic indicator of assisted reproductive technology outcome. *Fertil Steril*. 87, 93–100.
- Berlinguer, F., Madeddu, M., Pasciu, V., Succu, S., Spezzigu, A., Satta, V., Mereu, P., Leoni, G.G., Naitana, S., 2009. Semen molecular and cellular features: these parameters can reliably predict subsequent ART outcome in a goat model. *Reprod Biol Endocrinol*. 9, 125.
- Björndahl, L., Haugen, T.B., 2008. The information contained in a semen analysis. *Tidsskr Nor Laegeforen*. 128, 320–3.
- Bungum, M., Humaidan, P., Axmon, A., Spano, M., Bungum, L., Erenpreiss, J., Giwercman, A., 2007. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. *Hum Reprod*. 22, 174–179.
- Bungum, M., Bungum, L., Giwercman, A., 2011. Sperm chromatin structure assay (SCSA): a tool in diagnosis and treatment of infertility. *Asian J Androl*. 13, 69–75.
- Bungum, M., Humaidan, P., Spano, M., Jepson, K., Bungum, L., Giwercman, A., 2004. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod*. 19, 1401–8.
- Cates, W., Farley, T.M., Rowe, P.J., 1985. Worldwide patterns of infertility: is Africa different? *The Lancet*. 326, 596–598.
- Chohan, K.R., Griffin, J.T., Lafromboise, M., de Jonge, C.J., Carrell, D.T., 2006. Comparison of Chromatin Assays for DNA Fragmentation Evaluation in Human Sperm. *J Androl*. 27, 53–9.
- Claassens, O.E., Menkveld, R., Franken, D.R., Pretorius, E., Swart, Y., Lombard, C.J., Kruger, T.F., 1992. The Acridine Orange test: determining the relationship between sperm morphology and fertilization in vitro. *Hum Reprod*. 7, 242–247.
- Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivao, I., Giovannelli, L., Kruszewski, M., Smith, C.C., Stetina, R., 2008. The comet assay: topical issues. *Mutagenesis*. 23, 143–151.
- Das, S., Chattopadhyay, R., Ja, S.K., Babu, N.K., Chakraborty, C., Chakravarty, B., Chaudhury, K., 2008. Cut-off Value of Reactive Oxygen Species for Predicting Semen Quality and Fertilization Outcome. *Syst Biol Reprod Med*. 54, 47–54.
- Decordier, I., Vande Loock, K., Kirsch-Volders, M., 2010. Phenotyping for DNA repair capacity. *Mutat Res*. 705, 107–129.
- Devroey, P., Godoy, H., Smits, J., Camus, M., Tournaye, M., Derde, M-P., Van Steirteghem, A., 1996. Female age predicts embryonic implantation after ICSI: a case-controlled study. *Hum Reprod*. 11, 1324–1327.
- Dhawan, A., Bajpayee, M., Parmar, D., 2009. Comet assay: a reliable tool for the assessment of DNA damage in different models. *Cell Biol Toxicol*. 25, 5–32.
- Didion B, Kasperson K, Wixon R, *et al.*; Boar fertility and sperm chromatin structure status: A retrospective report. *J Androl*. 2009;30:655–660
- Donnelly, E.T., McClure, N., Lewis, S.E., 1999. The effect of ascorbate and alpha-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis*. 14, 505–12.
- Donnelly, E.T., Lewis, S.E., McNally, J.A., Thompson, W., 1998. In vitro fertilization and pregnancy rates: the influence of sperm motility and morphology on IVF outcome. *Fertil Steril*. 70, 305–14.
- du Plessis, S.S., Kashou, A.H., Benjamin, D.J., Yadav, S.P., Agarwal, A., 2011. Proteomics: a subcellular look at spermatozoa. *Reprod Biol Endocrinol*. 9–36.
- Dumoulin, J.C., Land, J.A., Van Montfort, A.P., Nelissen, E.C., Coonen, E., Derhaag, J.G., Schreurs, I.L., Dunselman, G.A., Kester, A.D., Geraedts, J.P., Evers, J.L., 2010. Effect of in vitro culture of human embryos on birthweight of newborns. *Hum Reprod*. 25, 605–612.
- Dupas, C., Christine-Maitre, S., 2008. What are the factors affecting fertility in 2008? *Ann Endocrinol*. 69, 57–61.
- Ebner, T., Shebl, O., Moser, M., Mayer, R.B., Arzt, W., Tews, G., 2011. Easy sperm processing technique allowing exclusive accumulation and later usage of DNA-strandbreak-free spermatozoa. *Reprod BioMed Online*. 22, 37–43.
- Eggert-Kruse, W., Rohr, G., Kerbel, H., Schwalbach, B., Demirakca, T., Klinga, K., Tilgen, W., Runnebaum, B., 1996. The acridine orange test: a clinically relevant screening method for sperm quality during infertility investigation. *Hum Reprod*. 11, 784–790.
- Evenson DP, Darzynkiewicz Z, and Melamed MR, Relation of mammalian sperm chromatin heterogeneity to fertility. *Science*. 1980;210:1131–1133.
- Evenson DP, Jost LK, Marshall, D., Zinaman MJ, Clegg, E., Purvis, K., de Angelis, P., Claussen., OP. 1999. Utility of the sperm chromatin structure assay (SCSA) as a diagnostic and prognostic tool in the human fertility clinic. *Human Reproduction*. 14(4):1039–1049.
- Ford, W.C.L., 2010. Comments on the release of the 5th edition of the WHO Laboratory Manual for the Examination and Processing of Human Semen. *Asian J Androl*. 12, 59–63.
- Freour, T., Delvigne, A., Barrière, P., 2010. Evaluation of the male of the infertile couple. *J Gynécol Obstét Biol Reprod*. 32, S45–52.

- Garrido, N., Remohí, J., Martínez-Conejero, J., García-Herrero, S., Pellicer, A., Meseguer, M., 2008. Contribution of sperm molecular features to embryo quality and assisted reproduction success. *Reprod Biomed Online*. 17, 855–65.
- Gharagozloo, P., Aitken, J.R., 2011. The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Hum Reprod*. 26, 1628–1640.
- Giwerzman A, Lindstedt L, Larsson M, Bungum M, Spano M, Levine RJ, *et al*. Sperm chromatin structure assay as an independent predictor of fertility in vivo: a case-control study. *Int J Androl* 2009;32:1–7.
- Gu, L.G., Chen, Z.W., Lu, W.H., Xu, J.F., Li, M., Chen, Z.J., 2011. Effects of abnormal structure of sperm chromatin on the outcome of in vitro fertilization and embryo transfer. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi*. 28, 156–9.
- Habbema, J.D., Eijkemans, M.J., Nargund, G., Beets, G., Leridon, H., te Velde, E.R., 2009. The effect of in vitro fertilization on birth rates in western countries. *Hum Reprod*. 24, 1414–1419.
- HFEA. A long term analysis of the HFEA Register data (1991–2006). Human Fertilisation and Embryology Authority. 2008.
- Host, E., Lindenberg, S., Smidt-Jensen, S., 2000. DNA strand breaks in human spermatozoa: correlation with fertilization in vitro in oligozoospermic men and in men with unexplained infertility. *Acta Obstet Gynecol Scand*. 79, 189–193.
- Host, E., Lindenberg, S., Smidt-Jensen, S., 2000. The role of DNA strand breaks in human spermatozoa used for IVF and ICSI. *Acta Obstet Gynecol Scand*. 79, 559–563.
- Hu, Y., Maxson, W.S., Hoffman, D.I., Ory, S.J., Eager, S., Dupre, J., Lu, C., 1998. Maximizing Pregnancy Rates and Limiting Higher-Order Multiple Conceptions by Determining the Optimal Number of Embryos to Transfer Based on Quality. *Fertil Steril*. 69, 650–657.
- Hughes, C.M., Lewis, S.E., McKelvey-Martin, V.J., Thompson, W., 1997. Reproducibility of human sperm DNA measurements using the alkaline single cell gel electrophoresis assay. *Mutat Res*. 374, 261–268.
- Hull, M.G., Glazener, C.M., Kelly, N.J., Conway, D.I., Foster, P.I., Hinton, R.A., 1985. Population study of causes, treatment and outcome of infertility. *BMJ*. 291, 1693–7.
- Human Fertilisation and Embryology Authority. Long-term trends in fertility treatment. 2008.
- Irvine, S.D., Twigg, J.P., Gordon, E.L., Fulton, N., Milne, P.A., Aitken, J.R., 2000. DNA integrity in human spermatozoa: relationships with semen quality. *J Androl*. 21, 33–44.
- Irvine, S.D., 1998. Epidemiology and aetiology of male infertility. *Hum Reprod*. 13, 33–44.
- ISO 5725:1994(E).
- Jha, A.N., 2008. Ecotoxicological applications and significance of the comet assay. *Mutagenesis*. 23, 207–221.
- Karlsson, H.L., 2010. The comet assay in nanotoxicology research. *Anal Bioanal Chem*. 398, 651–666.
- Kols, A., Nguyen, T., 1997. Infertility in Developing Countries. *Outlook*. 15.
- Koskimies, A.I., Savander, M., Ann-Marie, N., Kurunmäki, H., 2010. Sperm DNA damage and male infertility. *Duodecim*. 126, 2837–42.
- Lee, J.Y., Dada, R., Sabanegh, E., Carpi, A., Agarwal, A., 2010. Role of genetics in Azoospermia. *Infertility*. 598–601.
- Lee, T.-H., Chen, C.-D., Tsai, Y.-Y., Chang, L.-J., Ho, H.-N., Yang, Y.-S., 2006. Embryo quality is more important for younger women whereas age is more important for older women with regard to in vitro fertilization outcome and multiple pregnancy. *Fertil Steril*. 86, 64–69.
- Leroy, T., Van Hummelen, P., Anard, D., Castelain, P., Kirsch-Volders, M., Lauwerys, R., Lison, D., 1996. Evaluation of three methods for the detection of DNA single-strand breaks in human lymphocytes: alkaline elution, nick translation, and single-cell gel electrophoresis. *J toxicol environ health*. 47, 409–22.
- Lewis, S.E.M., 2007. Is sperm evaluation useful in predicting human fertility? *Reprod*. 134, 31–40.
- Lewis, S.E.M., Agbaje, I.M., 2008. Using the alkaline comet assay in prognostic tests for male infertility and assisted reproductive technology outcomes. *Mutagenesis*. 23, 163–170.
- Lewis, S.E.M., Simon, L., 2010. Clinical implications of sperm DNA damage. *Hum Fertil*. 13, 201–7.
- Lin, M.-H., Kuo-Kuang, Lee, R., Li, S.-H., Lu, C.-H., Sun, F.-J., Hwu, Y.-M., 2008. Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates in in vitro fertilization and intracytoplasmic sperm injection, but might be related to spontaneous abortion rates. *Fertil Steril*. 90, 352–359.
- Loi, K., Prasath, E.B., Huang, Z.W., Loh, S.F., Loh, S.K.E., 2008. A cumulative embryo scoring system for the prediction of pregnancy outcome following intracytoplasmic sperm injection. *SMJ*. 49, 221–227.
- Lombardo, F., Sansone, A., Romanelli, F., Paoli, D., Gandini, L., Lenzi, A., 2001. The role of antioxidant therapy in the treatment of male infertility: an overview. *Asian J Androl*. 13, 690–7.
- Mahfouz, R., Sharma, R., Thiagarajan, A., Kale, V., Gupta, S., Sabanegh, E., Agarwal, A., 2010. Semen characteristics and sperm DNA fragmentation in infertile men with low and high levels of seminal reactive oxygen species. *Fertil Steril*. 94, 2141–2146.
- Meseguer, M., Santiso, R., Garrido, N., Garcia-Herrero, S., Remohí, J., Fernandez, J.L., 2011. Effect of sperm DNA fragmentation on pregnancy outcome depends on oocyte quality. *Fertil Steril*. 95, 124–128.
- Meseguer, M., Martínez-Conejero, J.A., O'Connor, E.J., Pellicer, A., Remohí, J., Garrido, N., 2008. The significance of sperm DNA oxidation in embryo development and reproductive outcome in an oocyte donation program: a new model to study a male infertility prognostic factor. *Fertil Steril*. 89, 1191–1199.

- De Mouzon, J., Goossens, V., Bhattacharya, S., Castilla, J.A., Ferraretti, A.P., Korsak, V., Kupka, M., Nygren, K.G., Nyboe Andersen, A., 2010. Consortium The European IVF-monitoring (EIM). Assisted reproductive technology in Europe, 2006: results generated from European registers by ESHRE. *Hum Reprod.* 25, 1851–1862.
- Nagler, H.M., 2011. Male factor infertility: A solitary semen analysis can never predict normal fertility. *Nat Rev Urol.* 8, 16–17.
- Niederberger, C., Joyce, G.F., Wise, M., Meacham, R.B., 2007. Male infertility, in: Litwin MS, Saigal CS, editors. *Urologic Diseases in America.*, Washington, DC: US Government Printing Office.
- Pfuhler, S., Fellows, M., van Benthem, J., Corvi, R., Curren, R., Dearfield, K., Fowler, P., Frötschl, R., Elhajouji, A., Le Hégarat, L., Kasamatsu, T., Kojima, H., Ouédraogo, G., Scott, A., Speit, G., 2005. In vitro genotoxicity test approaches with better predictivity: Summary of an IWGT workshop. *Mutat Res.* 588, 88–105.
- Povey, A.C., Stocks, S.J., 2010. Epidemiology and trends in male subfertility. *Hum Fertil (Camb).* 13,182–8.
- Qian, Y.-I., Ye, Y.-H., Xu, C.-M., Jin, F., Huang, H.-F., 2008. Accuracy of a combined score of zygote and embryo morphology for selecting the best embryos for IVF. *J Zhejiang Univ Sci.* 9, 649–655.
- Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, Kirkman-Brown J, Coomarasamy A., 2012. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. *Hum Reprod.* 2012 Jul 12. [Epub ahead of print]
- Ross, C., Morriss, A., Khairy, M., Khalaf, Y., Braude, P., Coomarasamy, A., El-Toukhy, T.T., 2010. A systematic review of the effect of oral antioxidants on male infertility. *Reprod Biomed Online.* 20, 711–23.
- Rutstein, S.O., Shah, I.H., 2004. Infecundity, Infertility, and Childlessness. DHS Comparative Reports No. 9, DHS.
- Saleh, R.A., Agarwal, A., Nada, E.A., El-Tonsy, M.H., Sharma, R.K., Meyer, A., Nelson, D.R., Thomas, A.J., 2003. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril.* 79, 1597–605.
- Shamsi, M.B., Venkatesh, S., Tanwar, M., Singh, G., Mukherjee, S., Malhotra, N., Kumar, R., Gupta, N.P., Mittal, S., Dada, R., 2010. Comet assay: a prognostic tool for DNA integrity assessment in infertile men opting for assisted reproduction. *Indian J Med Res.* 131, 675–681.
- Simon, L., Lewis, S.E.M., 2011. Sperm DNA damage or progressive motility: which one is better predictor of fertilization in vitro? *Syst Biol Reprod Med.* 57, 133–138.
- Simon, L., Brunborg, G., Stevenson, M., Lutton, D., McManus, J., Lewis, S.E.M., 2010. Clinical significance of sperm DNA damage in assisted reproduction outcome. *Hum. Reprod.* 25, 1594–1608
- Simon, L., Lutton, D., McManus, J., Lewis, S.E.M., 2011. Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success. *Fertil Steril.* 95, 652–657.
- Sjöblom, P., Menezes, J., Cummins, L., Mathiyalagan, B., Costello, M.F., 2006. Prediction of embryo developmental potential and pregnancy based on early stage morphological characteristics. *Fertil Steril.* 86, 848–861.
- Speyer, B.E., Pizzey, A.R., Ranieri, M., Joshi, R., Delhanty, J.D.A., Serhal, P., 2010. Fall in implantation rates following ICSI with sperm with high DNA fragmentation. *Hum Reprod.* 25, 1609–1618.
- Stewart, L.M., Holman, C.D., Hart, R., Finn, J., Mai, Q., Preen, D.B., 2011. How effective is in vitro fertilization, and how can it be improved? *Fertil Steril.* 95, 1677–1683.
- Tam, W.H., Tsui, M.H., Lok, I.H., Yip, S.-K., Yuen, P.M., Chung, T.K., 2005. Long-term reproductive outcome subsequent to medical versus surgical treatment for miscarriage. *Hum Reprod.* 20, 3355–3359.
- Van Steirteghem, A., Liu, J.J., Joris, H.J., Nagy, Z.Z., Staessen, C., Camus, M., Wisanto, A., Van Assche, E., Devroey, P., 1993. Assisted fertilization by subzonal insemination and intracytoplasmic sperm injection. *Reprod Fertil Dev.* 6, 85–9.
- Vela, G., Luna, M., Sandler, B., Copperman, A.B., 2009. Advances and Controversies in Assisted Reproductive Technology. *Mt Sinai J Med.* 76, 506–520.
- Villani, P., Eleuteri, P., Grollino, M.G., Rescia, M., Altavista, P., Spano, M., Pacchierotti, F., Cordelli, E., 2010. Sperm DNA fragmentation induced by DNase I and hydrogen peroxide: an in vitro comparative study among different mammalian species. *Reproduction.* 140, 445–452.
- World Health Organization, 2010. ‘Mother or nothing: the agony of infertility.’ *Bulletin of the World Health Organization.* 88, 877–953.
- World Health Organization, Department of Reproductive Health and Research, 2010. WHO laboratory manual for the examination and processing of human semen. Fifth edition. WHO.
- Zini, A., 2011. Are sperm chromatin and DNA defects relevant in the clinic? *Syst Biol Reprod Med.* 57, 78–85.
- Zini, A., Jamal, W., Cowan, L., Al-Hathal, N., 2011. Is sperm dna damage associated with IVF embryo quality? A systematic review. *J Assist Reprod Genet.* 28,391–7.
- Zini, A., Al-Hathal, N., 2011. Antioxidant therapy in male infertility: fact or fiction? *Asian J Androl.* 13, 374–381.
- Zini, A., Boman, J.M., Belzile, E., Ciampi, A., 2008. Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. *Hum Reprod.* 23, 2663–2668.
- Zribi, N., Chakroun, N.F., Elleuch, H., Abdallah, F.B., Ben Hamida, A.S., Gargouri, J., Fakhfakh, F.F., Keskes, L.A., 2011. Sperm DNA fragmentation and oxidation are independent of malondialdehyde. *Reprod Biol Endocrinol.* 9, 1–8.

Declaration: IP is an employee and a shareholder of Lewis Fertility Testing, a spin-out company of Queen's University Belfast, which is now marketing the Comet test. SEMI is the Chief Executive Officer and a shareholder of Lewis Fertility Testing. The other authors report no financial or commercial conflicts of interest.

ACCEPTED MANUSCRIPT

Table 1. Demographic characteristics of IVF patients.

<i>Characteristic</i>	<i>Total</i>	<i>Idiopathic infertility</i>	<i>Live birth</i>	<i>Miscarriage</i>			<i>P-value</i>	<i>No pregnancy</i>		
				<i>Mean</i>	<i>95% CI</i>			<i>Mean</i>	<i>95% CI</i>	<i>P-value</i>
No. of cycles	203	70	41	9				153		
Female age (years)	35.6 ± 0.3	36.3 ± 0.4	34.5 ± 0.7	34.2 ± 1.0	-3.282 to 2.682	NS		35.9 ± 0.3	-2.713 to 0.08691	0.04
Male age (years)	37.1 ± 0.4	37.2 ± 0.6	35.5 ± 0.9	36.4 ± 2.1	-2.760 to 4.560	NS		37.5 ± 0.4	-3.647 to 0.3526	0.02
No. of treatments	1.4 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.4 ± 0.2	-0.7444 to 0.5444	NS		1.4 ± 0.1	-0.2378 to 0.4378	NS
Oocytes fertilized (2 pronucleus)	5.0 ± 0.3	4.9 ± 0.4	6.9 ± 0.6	7.4 ± 1.5	-2.172 to 3.172	NS		4.4 ± 0.3	1.321 to 3.679	< 0.0001
Embryos transferred	1.8 ± 0.04	1.8 ± 0.1	1.9 ± 0.04	2.0 ± 0.0	-0.03529 to 0.2353	NS		1.8 ± 0.05	-0.05654 to 0.2565	NS
Total embryo cumulative score	13.9 ± 0.7	12.6 ± 1.1	14.5 ± 1.1	19.3 ± 3.2	0.8416 to 8.758	0.02		13.4 ± 0.9	-1.316 to 3.516	NS
Transferred embryo cumulative score	37.8 ± 1.4	36.1 ± 2.2	43.0 ± 2.7	49.3 ± 5.7	-3.714 to 16.31	NS		35.8 ± 1.7	1.702 to 12.70	0.01

Values are mean ± SE unless otherwise stated.
P-values are for comparison with live births.
NS = not significant.

Table 2. Demographic characteristics of ICSI patients.

<i>Characteristic</i>	<i>Total</i>	<i>Live birth</i>	<i>Miscarriage</i>		<i>No pregnancy</i>	
			<i>Mean</i>	<i>95% CI</i>	<i>Mean</i>	<i>95% CI</i>
No. of cycles	136	34	4		99	
Female age (years)	34.9 ± 0.4	34.5 ± 0.6	35.5 ± 1.7	−3.034 to 5.034	35 ± 0.4	−2.128 to 1.128
Male age (years)	37.2 ± 0.4	37.5 ± 0.7	38.5 ± 2.7	−3.341 to 5.341	36.9 ± 0.6	−1.128 to 2.328
No. of treatments	1.7 ± 0.1	1.6 ± 0.1	1.5 ± 0.3	−0.9424 to 0.7424	1.7 ± 0.1	−0.4715 to 0.2715
Oocytes fertilized (2 pronucleus)	5.3 ± 0.3	6.1 ± 0.6	6.5 ± 1.9	−3.040 to 3.840	4.9 ± 0.3	0.05690 to 2.343 ^a
Embryos transferred	1.8 ± 0.03	1.9 ± 0.04	2.0 ± 0.0	−0.1054 to 0.3054	1.8 ± 0.0	−0.04035 to 0.2404
Total embryo cumulative score	13.2 ± 0.9	12.8 ± 1.6	14.9 ± 0.05	−3.754 to 7.954	13.3 ± 1.1	−2.663 to 1.663
Transferred embryo cumulative score	38.2 ± 2.7	38.8 ± 4.7	48.0 ± 16.0	−9.706 to 28.11	37.1 ± 3.3	−4.759 to 8.159

Values are mean ± SE unless otherwise stated.

^a*P* = 0.04 for comparison with live births.

Table 3. Pregnancy, miscarriage and live-birth rates for men, categorized by sperm DNA damage in native semen.

Treatment	Pregnancies	Miscarriages	Live births	<25% sperm DNA damage				25–50% sperm DNA damage				>50% sperm DNA damage			
				Total	CP	M	LB	Total	CP	M	LB	Total	CP	M	LB
IVF (n = 203)	50 (24.6)	9 (4.4)	41 (20.2)	33 (16.3)	13 (9.4) ^a	2 (1.1)	11 (3.3) ^b	7 (3.5)	21 (9.6) ^a	4 (5.6)	17 (9.9) ^b	99 (48.8)	16 (6.2) ^a	3 (3.3)	13 (3.1) ^b
ICSI (n = 136)	38 (27.9)	4 (2.9)	34 (25.0)	9 (6.6)	2 (2.2)	0 (0)	2 (2.2)	3 (2.5)	13 (8.2)	2 (5.9)	11 (3.4)	93 (68.4)	21 (22.6)	2 (2.2)	19 (20.4)

Values are n (%). Sperm DNA fragmentation thresholds as published in Simon *et al.* (2010, 2011).

^aP-value for pregnancy rate = 0.005.

^bP-value for live-birth rate = 0.007.

CP = clinical pregnancy rate; ICSI = intracytoplasmic sperm injection; LB = live-birth rate; M = miscarriage rate.

Table 4. Sperm DNA fragmentation in men and couples with idiopathic infertility.

<i>Group with idiopathic infertility</i>	<i>Native semen</i>	<i>Prepared spermatozoa</i>
Men (<i>n</i> = 147)	46.9 ± 1.8	32.8 ± 1.6
Couples (<i>n</i> = 70)	45.8 ± 2.6	32.7 ± 2.4

Values are mean ± SE.

Table 5. Outcomes in men and couples with idiopathic infertility, categorized by sperm DNA damage in native semen.

<i>Group with idiopathic infertility</i>	<i>Pregnancies</i>	<i>Miscarriages</i>	<i>Live births</i>	<i><25% sperm DNA damage</i>				<i>25–50% sperm DNA damage</i>				<i>>50% sperm DNA damage</i>			
				<i>Total</i>	<i>CP</i>	<i>M</i>	<i>LB</i>	<i>Total</i>	<i>CP</i>	<i>M</i>	<i>LB</i>	<i>Total</i>	<i>CP</i>	<i>M</i>	<i>LB</i>
Men (n = 147)	31 (21.1)	6 (4.1)	25 (17.0)	23 (15.6)	6 (26.1)	0 (0)	6 (26.1)	64 (43.5)	16 (25.0)	4 (6.3)	12 (18.8)	60 (40.8)	9 (15.0)	2 (3.3)	7 (11.7)
Couples (n = 70)	14 (20.0)	4 (5.7)	10 (14.3)	11 (15.7)	3 (27.3)	0 (0)	3 (27.3)	32 (45.7)	7 (21.9)	3 (9.4)	4 (12.5)	27 (38.6)	4 (14.8)	1 (3.7)	3 (11.1)

Values are *n* (%). Sperm DNA fragmentation thresholds as published in Simon *et al.* (2010, 2011).
CP = clinical pregnancy rate; LB = live-birth rate; M = miscarriage rate.

Figure 1. Sperm DNA damage in patients undergoing IVF or intracytoplasmic sperm injection (ICSI) as assessed by the alkaline Comet assay. Each group was compared with the live-birth group and confidence intervals and *P*-values were calculated for each comparison. Bar = mean \pm SE. **P* < 0.05; ***P* < 0.01.

Figure 2. Normal Q–Q plot of the sperm DNA damage residual. Residuals were plotted for all means.

Figure 1. Sperm DNA damage in patients undergoing IVF or ICSI treatment as assessed by the alkaline Comet assay. Each group was compared to the “live birth” group and confidence intervals and a P values were calculated for each comparison. Each bar represents means \pm SE. *P<0.05, **P<0.01.

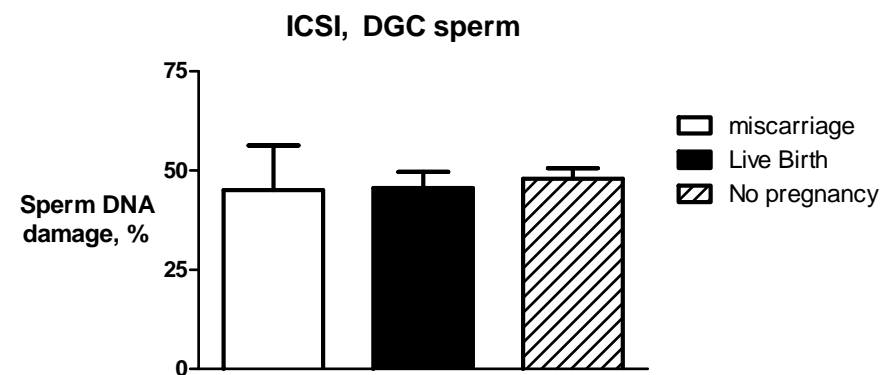
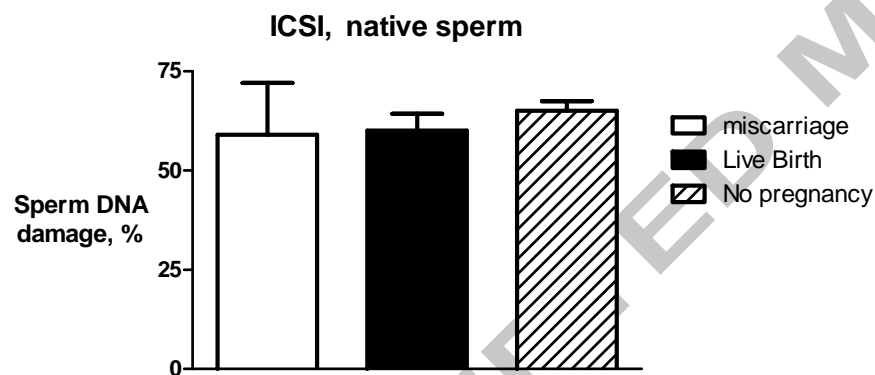
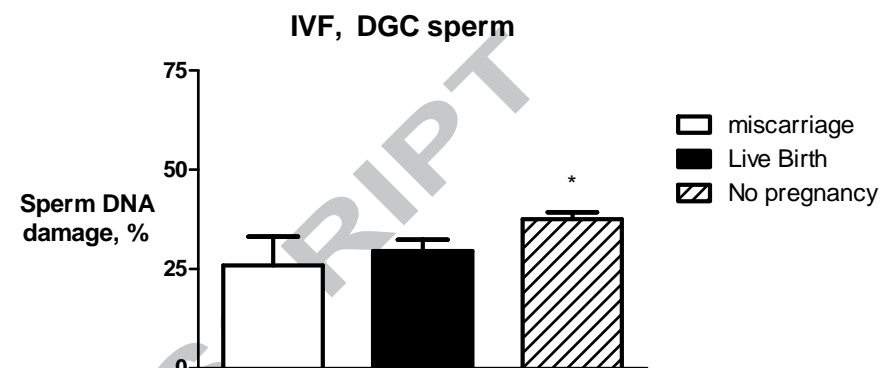
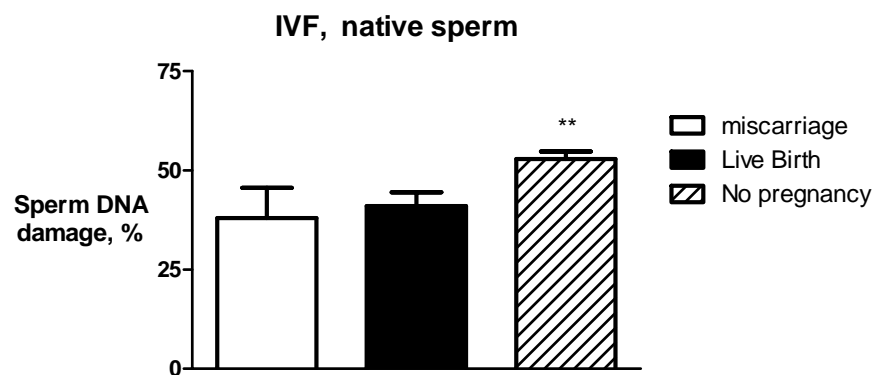
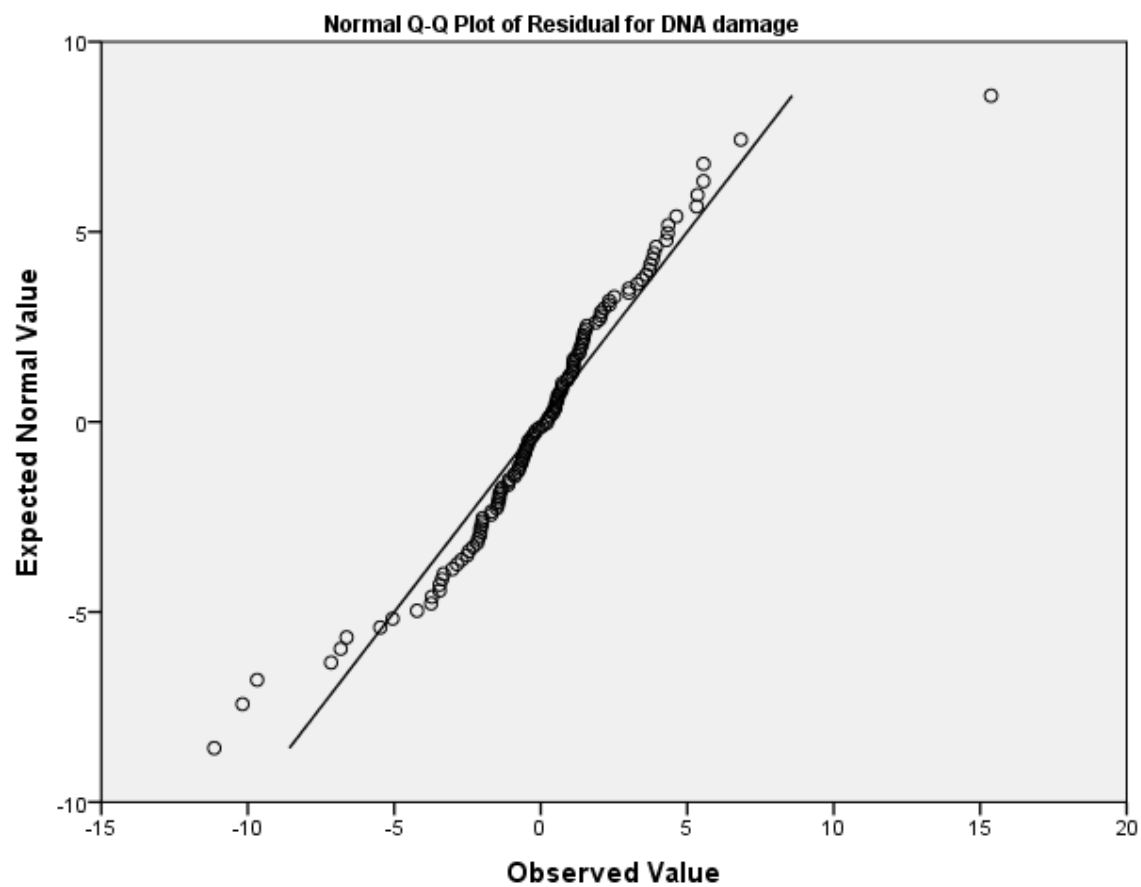


Figure 2. Normal Q_Q plot of the DNA damage residual. Residuals were plotted for all DNA damage means.



Sperm DNA damage has a negative impact on assisted reproduction treatment outcome, in particular, on pregnancy rates. The aim of the present study was to examine the relationship between sperm DNA fragmentation and live-birth rates after IVF and intracytoplasmic sperm injection (ICSI). The alkaline Comet assay was employed to measure sperm DNA fragmentation in native semen and in spermatozoa following density-gradient centrifugation in semen samples from 203 couples undergoing IVF and 136 couples undergoing ICSI. Men were divided into groups according to sperm DNA damage and treatment outcome. Following IVF, couples with <25% sperm DNA fragmentation had a live birth rate of 33%. In contrast, couples with >50% sperm DNA fragmentation had a much lower live-birth rate of 13% following IVF. Following ICSI, there were no significant differences in levels of sperm DNA damage between any groups of patients. Sperm DNA damage was also associated with the very low live-birth rates following IVF in both men and couples with idiopathic infertility: 39% of couples and 41% of men have high level of sperm DNA damage. Sperm DNA damage assessed by the Comet assay has a close inverse relationship with live-birth rates after IVF.

Luke Simon graduated in biotechnology from the University of Madras, India in 2005 with a specialization in DNA fingerprinting. From 2005 to 2007, he was an instructor and research assistant at the University of Agricultural Sciences, Bangalore, India, during which he explored various types of DNA markers to identify inter- and intra-species variations. In 2007, he joined the Centre for Public Health at Queen's University Belfast as a doctoral research fellow. He is now a postdoctoral researcher at the School of Medicine, University of Utah, USA. His principal interests focus on sperm DNA damage, protamine and the effects of oxidative stress on male fertility.

